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A mixture of 15 phthalates and pesticides below individual chemical no observed adverse effect levels (NOAELs) produces reproductive tract malformations in the male rat

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Background/Overview: Humans carry residues of multiple synthetic chemicals at any given point in time. Research has demonstrated that compounds with varying molecular mechanisms that disrupt common Key Events can act in concert to produce cumulative adverse effects. As such, one of the most pressing issues in toxicology and risk assessment is the evaluation and cumulative assessment of chemical mixtures. Our research group has published several research studies on the combined effects of *in utero* exposure to chemicals that have converging Key Events within an androgen receptor signaling Adverse Outcome Pathway network. This manuscript describes recent experiments within this line of inquiry with a study design based on individual chemical no observed adverse effect levels (NOAELs). We hypothesized that a broad mixture (15 compounds) of phthalates and pesticides would produce permanent, adverse male reproductive effects when each chemical was administered at or below a dose at which the individual chemicals produce no effects in isolation (i.e., below NOAELs).

Relevance to EPA Program/Regional Research Needs/Priorities: This paper is identified as subproduct CSS.4.2.1.1 and is relevant to the needs of OPPT as they consider a phthalate cumulative risk assessment approach. Further, one of the highest priorities in EPA is the advancement of methods and scientific rationale for addressing issues related to chemical mixtures.

Name(s) of Program Office Reviewer(s) of Earlier Drafts: OPPT/ECRAD scientist Dr. Anthony Luz provided a Technical Manuscript Review.

Program Office/Regional Office Co-authors: None

Study Description: We conducted experiments with pregnant female rats to assess effects on androgen signaling and reproductive tract development in offspring at fetal, newborn, juvenile, and adult time points. Pregnant female rats were dosed during the "masculinizing window" of development, when the male reproductive tract is being programmed. We dosed the animals with vehicle control or dilutions of a mixture of 15 different anti-androgenic compounds including 6 pesticides and 9 phthalate plasticizers. The top dose (100% dose) contained each compound at a concentration 2-fold greater than the individual chemical NOAEL followed by a dilution series that represented each chemical at NOAEL, NOAEL/2, NOAEL/4, NOAEL/8, NOAEL/15, NOAEL/100, and NOAEL/1000 dose levels. Importantly, the 15 chemicals target at least 3 different molecular initiating events (i.e., mechanisms of action), but all of which ultimately disrupt androgen receptor signaling in the developing fetus.

Major Observations and Results: We found that a complex mixture of 15 anti-androgenic phthalates and pesticides with at least 3 different MIEs produced permanent malformations in F1 male reproductive tissues when each chemical was present at a dose which had been individually shown to produce no adverse male reproductive effects. Adverse outcomes including hypospadias and weight deficits in multiple accessory sex tissues (LABC, seminal vesicles, glans penis) occurred at doses 2-fold below individual NOAELs. Delayed puberty and biomarkers of effect including feminized anogenital distance and retention of female-like nipples/areolae occurred at doses 4-fold below NOAELs. Critical key events like reduced fetal testis testosterone production and gene expression, although not universally affected by all mixture chemicals, were affected at doses 8-fold and 15-fold below individual NOAELs, respectively. Chemical co-exposure reduced risk assessment-relevant ED10 estimates for hypospadias incidence ~25-fold, on average, for each chemical, demonstrating the potential importance of cumulative assessment approaches when estimating points-of-departure. Dose addition modeling more accurately predicted the dose response curves for three observed effects (reduced AGD, reduced seminal vesicle weight, and increased hypospadias), whereas response addition and integrated addition mildly or substantially underpredicted mixture potency.

Impact/Potential Implication of the Findings: This paper demonstrates the importance of assessing the toxicity for multiple chemicals with overlapping Key Events in an AOP network. We found that when individual chemicals that disrupt androgen receptor signaling via multiple MIEs are administered as a mixture to pregnant female rats during the critical window of fetal development of the reproductive tract, permanent adverse effects occur in the offspring. Critically, these effects occur at doses lower than those at which the individual chemicals have been shown to produce effects in individual exposures.

Findings Advancing Existing Scientific Knowledge: The present study suggests that male fetuses may be at increased risk due to cumulative exposure of the pregnant woman to multiple anti-androgenic chemicals, even when individual doses are below known levels of concern. Moving forward, cumulative risk assessment approaches should consider grouping chemicals based on a common biological signaling pathway and these pathways can be elucidated using the AOP framework. Further, the dose addition continues to be the most accurate model for predicting mixture-based toxicological effects.

Publication Information (journal, book chapter/book) and Estimated Timelines: This manuscript will be submitted to Environment International (Impact Factor: 7.943, open-access) for publication consideration. Depending on the length of time for peer review and revision, the manuscript could be expected to be available online within 2-4 months of submission and in print within 6-8 months.

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